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Effect of Polyamines on the Activity of Bovine Pancreatic Ribonuclease A using Cyclic Nucleotides, Nucleotide Benzyl Esters, and Polynucleotides as Substrates[1]

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Spermine stimulated the hydrolysis of 2′,3′-cyclic phosphates of cytidine and uridine by bovine pancreatic RNase A, the degree of stimulation being considerably greater with the former substrate. Similar effects of spermine were obtained with benzyl esters of 3′-cytidylic acid and 3′-uridylic acid as substrates. The degree of stimulation by spermine of the breakdown of poly(C) decreased with decrease in the molecular weight of poly(C), while the degradation of poly(U) was not influenced significantly by spermine in the molecular weight range of poly(U) from 30000 to 85000.

Kinetic studies showed that polyamines did not significantly change the apparent *K*m of the enzyme for any substrate tested, but increased the maximal velocity of the reaction when stimulation was observed.

Monovalent cations (NH_4^+ and K^+) had stimulatory effects similar to those of polyamines on RNase A activity towards cyclic nucleotides and polynucleotides.

Keywords——polyamines; bovine pancreatic ribonuclease A; cyclic nucleotides; nucleotide benzyl esters; polynucleotides; base specificity; spermine

Levy *et al.*[3] recently reported that polyamines can stimulate the activity of a ribonuclease from *Citrobacter* species and alter the ribonuclease base specificity. We have also reported that polyamines change the base specificity of bovine pancreatic RNase A and RNase HS from horse submaxillary gland, which hydrolyze RNA at pyrimidine nucleotide linkages.[4] In the presence of polyamines, the cleavage of $C_{5'}$-O-P linkages adjacent to a cytosine nucleotide was stimulated, while the cleavage of $C_{5'}$-O-P linkages adjacent to a uracil nucleotide was inhibited slightly.[4a]

In this paper we report the effect of spermine on the activity of RNase A using 2′,3′-cyclic nucleotides, 3′-nucleotide benzyl esters, and homopolynucleotides of different molecular weights as substrates, since it is of interest to know whether or not polyamines change the base specificity of RNase A even when small molecules are used as substrates. In addition, the effect of spermine on RNase A activity is compared with that of other cations in order to learn whether the function of polyamines is unique.

Materials and Methods

Materials——RNase A (type II-A) was purchased from Sigma Chemical Co. Yeast RNA (Nutritional Biochemical Co.) was used after purification by acid precipitation and dialysis against distilled water. Poly-

1) Abbreviations: RNase A, bovine pancreatic ribonuclease A; BSA, bovine serum albumin; 3′-CMP and 3′-UMP, 3′-phosphates of cytidine and uridine, respectively; 2′,3′-cCMP and 2′,3′-cUMP, 2′,3′-cyclic phosphates of cytidine and uridine, respectively; C-3′-*p*-benz and U-3′-*p*-benz, benzyl esters of 3′-cytidylic acid and 3′-uridylic acid, respectively.

2) Location: *1-33 Yayoi-cho, Chiba 260, Japan.*

3) C.C. Levy, W.E. Mitch, and M. Schmukler, *J. Biol. Chem.*, **248**, 5712 (1973); C.C. Levy, P.A. Hieter, and S.M. LeGendre, *ibid.*, **249**, 6762 (1974); J.J. Frank and C.C. Levy, *ibid.*, **251**, 5745 (1976).

4) *a*) K. Igarashi, H. Kumagai, Y. Watanabe, N. Tcyoda, and S. Hirose, *Biochem. Biophys. Res. Commun.*, **67**, 1070 (1975); *b*) K. Igarashi, Y. Watanabe, H. Kumagai, N. Ishizaki, and S. Hirose, *J. Biochem.*, **81**, 579 (1977).

(C) and poly(U) were purchased from Boehringer Mannheim GmbH. Cyclic nucleotides were prepared by the method of Smith *et al.*[5] and 3′-nucleotide benzyl esters were prepared by the procedure of Molemans *et al.*[6] Putrescine dihydrochloride, spermidine trihydrochloride, and spermine tetrahydrochloride were obtained from Nakarai Chemicals. The sodium salt of heparin (130 units/mg) was obtained from Daiichi Pure Chemicals Co.

Assay of RNase Activity on Cyclic Nucleotides——This was performed by the method of Richards[7] with a slight modification. The reaction mixture (1.5 ml), containing 0.2 mM 2′,3′-cCMP or 2′,3′-cUMP, 30 mM Tris–HCl (pH 8.2), 100 μg of BSA, and enzyme, was incubated at 37° for 20 hr. After the incubation, the absorbance at 285 nm (2′,3′-cCMP) or 280 nm (2′,3′-cUMP) was measured to calculate the amount of hydrolyzed product. The molecular extinction coeffecients of 2′,3′-cCMP and 2′,3′-cUMP at 260 nm were calculated to be 7840 and 9570, respectively, at pH 8.2 from the data of Brown *et al.*[8]

Assay of RNase Activity towards Nucleotide Benzyl Esters——This was performed as described previously,[9] except that the reaction mixture (3.0 ml), which contained 0.2 mM C-3′-*p*-benz or U-3′-*p*-benz, 30 mM Tris–HCl (pH 8.2), 20 μg of BSA, and enzyme, was incubated at 37° for 20 hr.

Assay of RNase Activity on Polynucleotides——The method used was essentially that of Anfinsen *et al.*[10] The standard assay system (0.2 ml) contained 100 μg of synthetic polynucleotide, 20 μg of BSA, 10 μmol of Tris–HCl (pH 8.2), and enzyme. After incubation at 37° for 15 min, the reaction was terminated by the addition of 0.2 ml of 5% perchloric acid containing 0.25% uranyl acetate. After being cooled in an ice-water bath for 30 min, the mixture was centrifuged and the resulting supernatant was diluted with 4 volumes of water. The amounts of acid-soluble nucleotides therein were measured in terms of the absorbance at 260 nm.

Results

Effect of Spermine on RNase A Activity using Cyclic Nucleotides and Nucleotide Benzyl Esters as Substrates

As shown in Fig. 1, the hydrolysis of both 2′,3′-cCMP and 2′,3′-cUMP by RNase A was stimulated by the addition of spermine. The degree of stimulation of the hydrolysis of 2′,3′-cCMP was considerably greater than that of 2′,3′-cUMP. The concentration of spermine required for maximal stimulation of the hydrolysis of the cyclic nucleotides increased as the substrate concentrations were increased (Fig. 2).

We have reported that the inhibition of RNase A activity by heparin was greater with poly(U) than with poly(C) as a substrate, and that the restoration of RNase activity by spermine was more efficient when poly(C) was used as a substrate in the presence of heparin.[4b] When cyclic nucleotides were used as substrates, the inhibition of RNase A activity by heparin was slightly greater with 2′,3′-cUMP than with 2′,3′-cCMP as a substrate (Figs. 1 and 3). Spermine restored the hydrolysis of 2′,3′-cCMP more efficiently than the hydrolysis of 2′,3′-cUMP (Fig. 3). Spermidine and putrescine showed effects similar to that of spermine on the hydrolysis of 2′,3′-cCMP by RNase A, although the effective concentrations were higher (Fig. 4).

We next studied the effect of spermine on the transphosphorylation reaction of RNase A using nucleotide benzyl esters as substrates. The transphosphorylation of C-3′-*p*-benz and U-3′-*p*-benz by RNase A was stimulated by spermine, the degree of stimulation being greater with the former substrate (Fig. 5). The effective concentrations of spermine required for stimulation of the transphosphorylation of the benzyl esters were higher when larger amounts of substrates were used (data not shown).

5) M. Smith, J.G. Moffatt, and H.G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958).
6) F. Molemans, M. van Montagu, and W. Fiers, *Eur. J. Biochem.*, **4**, 524 (1968).
7) F.M. Richards, *Compt. rend. trav. lab. Carlsberg Ser. chim.*, **29**, 315 (1955).
8) C.M. Brown, D.I. Magrath, and. A.R. Todd, *J. Chem. Soc.*, **1952**, 2708.
9) S. Hirose, H. Kumagai, M. Yoshikawa, T. Mikami, and K. Igarashi, *J. Biochem.*, **82**, 1605 (1977).
10) C.B. Anfinsen, R.R. Redfield, W.L. Choate, J. Page, and W.R. Carroll, *J. Biol. Chem.*, **207**, 201 (1954).

Effect of Spermine on the Activity of RNase A using Pyrimidine Homopolymers of Different Molecular Weights as Substrates

The effect of spermine was examined using homopolymers of different molecular weights. The degree of stimulation by spermine of the breakdown of poly(C) decreased with decrease

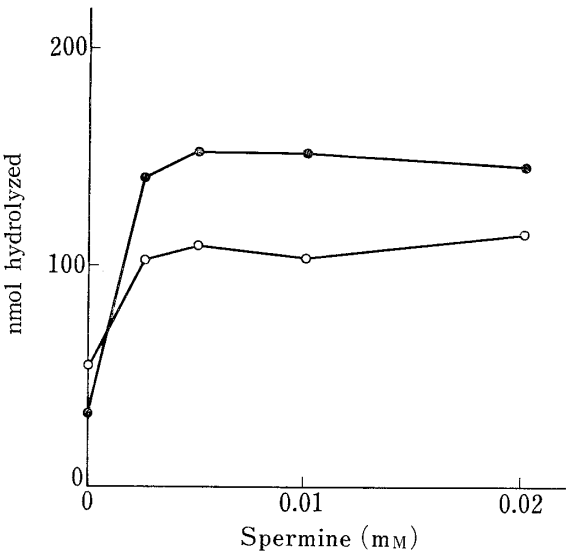

Fig. 1. Effect of Spermine on RNase A Activity using Cyclic Nucleotides as Substrates

The assays were carried out under standard conditions except that the amounts of RNase A used for the hydrolysis of 2′,3′-cCMP (●—●) and 2′,3′-cUMP (○—○) were 0.7 and 2.1 μg, respectively, and the reaction mixture contained spermine as specified in the figure.

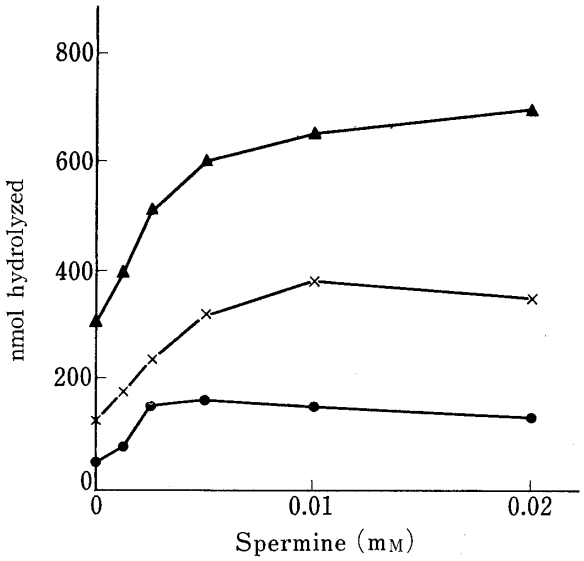

Fig. 2. Effect of 2′,3′-cCMP Concentration on the Stimulation of RNase A Activity by Spermine

The assays were carried out under standard conditions except that the reaction mixture contained 0.7 μg of RNase A and the specified concentrations of spermine and 2′,3′-cCMP.
●—●, 0.2 mM 2′,3′-cCMP; ×—×, 0.4 mM 2′,3′-cCMP; ▲—▲, 0.8 mM 2′,3′-cCMP.

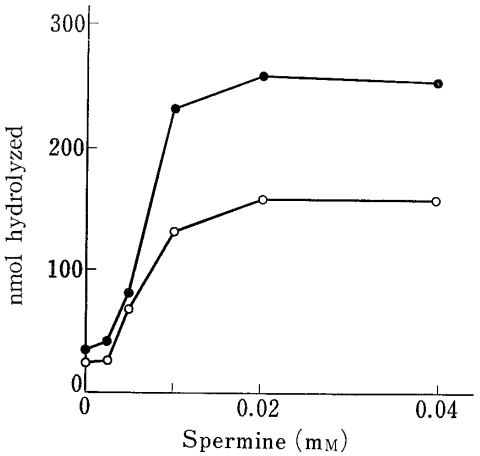

Fig. 3. Effect of Spermine on RNase A Activity using Cyclic Nucleotides as Substrates in the Presence of Heparin

The assays were carried out under standard conditions except that the amounts of RNase A used for the hydrolysis of 2′,3′-cCMP (●—●) and 2′,3′-cUMP (○—○) were 1.2 and 3.6 μg, respectively, and the reaction mixture contained 7.5 μg of heparin and spermine as specified in the figure.

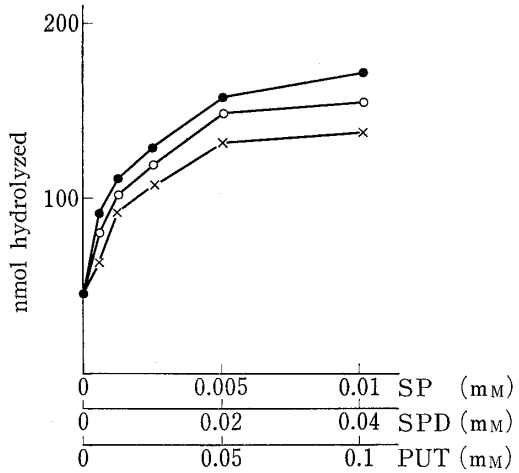

Fig. 4. Effect of Polyamines on RNase A Activity using 2′,3′-cCMP as a Substrate

The assays were carried out under standard conditions except that the reaction mixture contained 0.7 μg of RNase A, 0.2 mM 2′,3′-cCMP, and polyamines as specified in the figure.
●, spermine (SP); ○, spermidine (SPD); ×, putrescine (PUT).

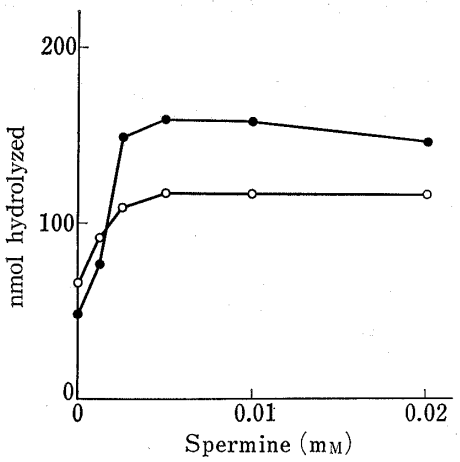

Fig. 5. Effect of Spermine on RNase A Activity using Nucleotide Benzyl Esters as Substrates

The assays were carried out under standard conditions except that the amounts of RNase A used for the hydrolysis of C-3′-*p*-benz (●—●) and U-3′-*p*-benz (○—○) were 1.2 and 3.6 μg, respectively, and the reaction mixture contained spermine as specified in the figure.

hibition by Mn^{2+} was very marked. similar to those of polyamines on RNase A activity.

in the molecular weight of poly(C) (Fig. 6A), while the degradation of poly(U) was not influenced significantly by the molecular weight of poly(U) in the range from 30000 to 85000 (Fig. 6B).

Effect of Monovalent and Divalent Cations on RNase A Activity using Cyclic Nucleotides and Polynucleotides as Substrates

In order to learn whether the change of base specificity of RNase A by polyamines is unique to this type of cation, the effects of monovalent and divalent cations on RNase A activity were studied. As shown in Fig. 7, NH_4^+ and K^+ stimulated both poly(C) breakdown and the hydrolysis of 2′,3′-cCMP, but Na^+ stimulated the hydrolysis of 2′,3′-cCMP only. The breakdown of poly(U) was inhibited slightly by NH_4^+, K^+, and Na^+, but the hydrolysis of 2′,3′-cUMP was stimulated slightly by these cations.

Although the hydrolysis of cyclic nucleotides by RNase A was stimulated by Mg^{2+}, Ca^{2+}, and Mn^{2+}, the breakdown of poly(U) and poly(C) was inhibited by Mg^{2+}, Ca^{2+}, and Mn^{2+} (data not shown). The inThese results suggest that NH_4^+ and K^+ have effects

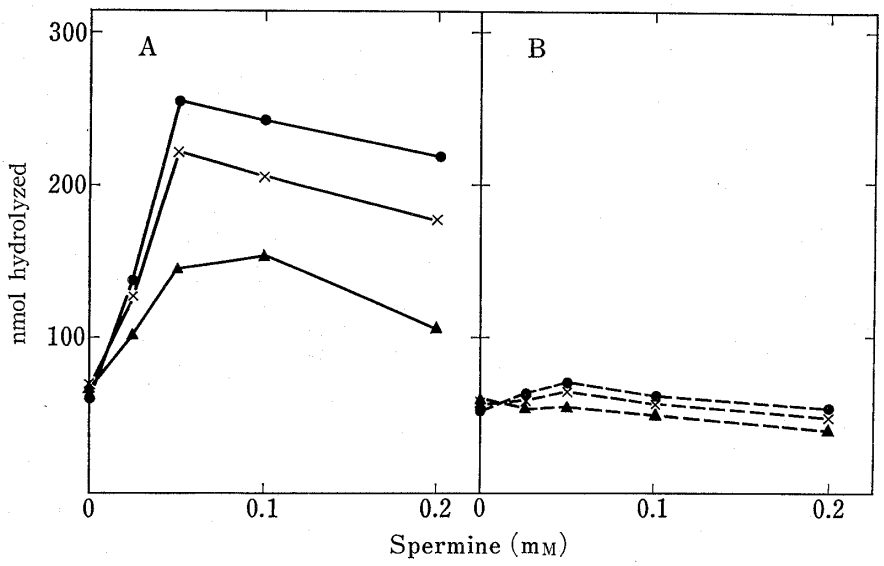

Fig. 6. Effect of Spermine on RNase A Activity towards Homopolynucleotides of Different Molecular Weights

The assays were carried out under standard conditions except that the reaction mixture contained spermine as specified in the figure. The amounts of RNase A used in A (poly(C)) and B (poly(U)) were 1 and 8 ng, respectively. To obtain preparations with different molecular weights, poly(C) and poly(U) were fractionated on a Sephadex G-75 column (4.2×91 cm) equilibrated and eluted with a buffer containing 10 mM Tris-HCl (pH 7.4) and 0.1 M NaCl. The molecular weight was estimated by the method of Andrews[a] using *Escherichia coli* tRNA (molecular weight: 25000), *E. coli* 5S RNA (molecular weight: 40000), and globin mRNA (molecular weight: 190000) as markers. Average molecular weights of polynucleotides were 85000 (●), 60000 (×), and 30000 (▲).

a) P. Andrews, *Biochem. J.*, **91**, 222 (1964).

Kinetic Studies to determine the Mode of Action of Polyamine Stimulation of RNase A Activity

The mechanism of stimulation of RNase A activity by spermine was analyzed by means of double-reciprocal plots. As shown in Figs. 8 and 9, spermine did not significantly change the apparent Km of RNase A. The Km values of RNase A for 2′,3′-cCMP, 2′,3′-cUMP, poly-

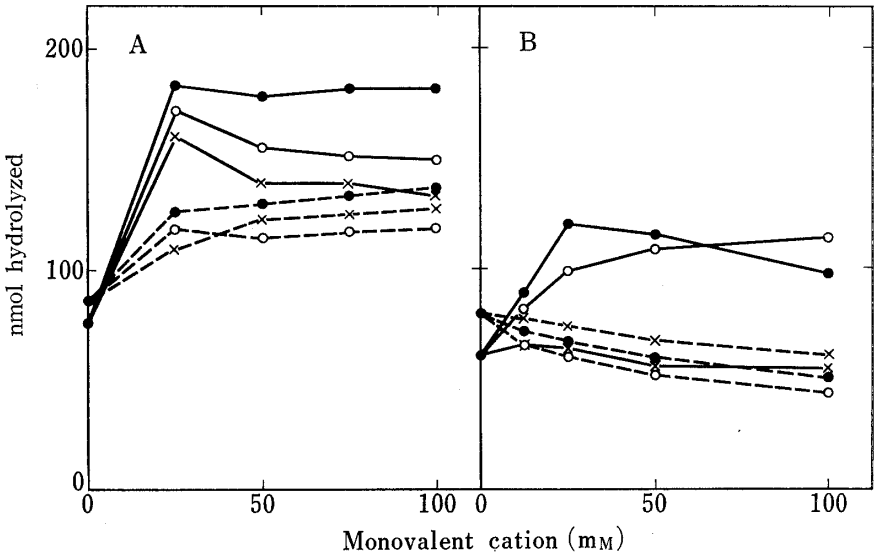

Fig. 7. Effects of Monovalent Cations on RNase A Activity towards Cyclic Nucleotides and Polynucleotides

The assays were carried out under standard conditions except that the reaction mixture contained monovalent cations as specified in the figure. The average molecular weight of polynucleotides used was 60000.
A (———), 0.2 mM 2′,3′-cCMP; A (– – – –), 0.2 mM 2′,3′-cUMP;
B (———), 100 μg of poly(C); B (– – – –), 100 μg of poly(U).
●, NH_4^+; ○, K^+; ×, Na^+.

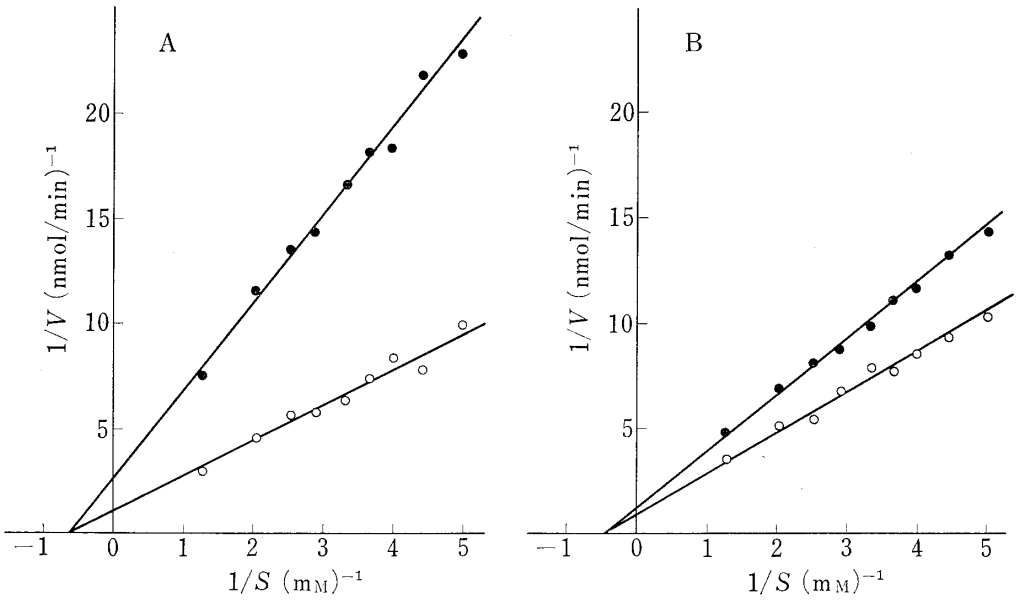

Fig. 8. Kinetics of the Hydrolysis of Cyclic Nucleotides by RNase A in the Presence and Absence of Spermine

The assays were carried out under standard conditions except that the amounts of RNase A used for the hydrolysis of 2′,3′-cCMP (A) and 2′,3′-cUMP (B) were 1 and 3 μg, respectively, and the reaction mixture contained various amounts of substrate and spermine as specified in the figure.
●—●, no spermine; ○—○, 0.02 mM spermine.

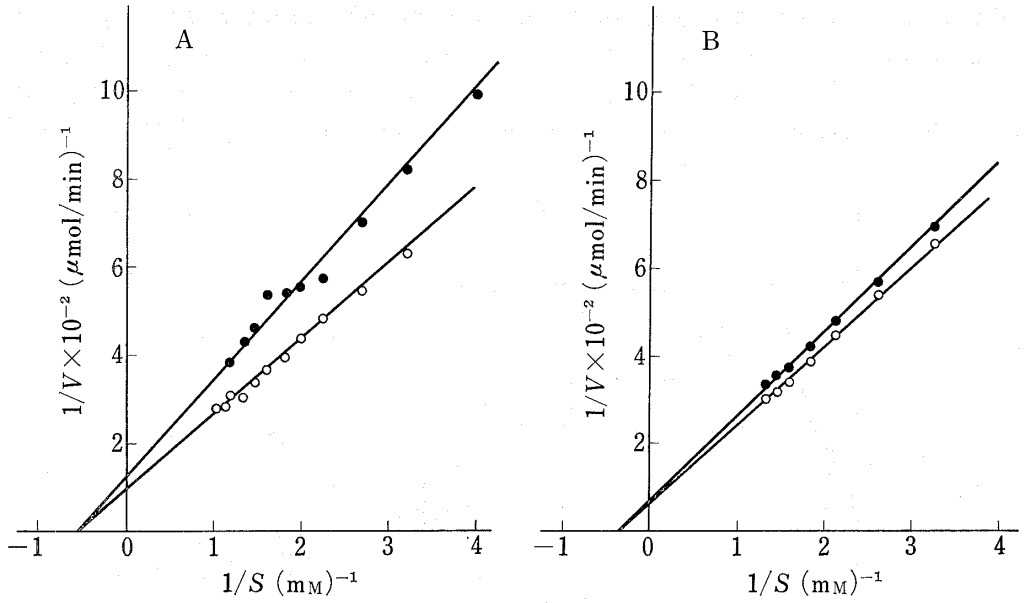

Fig. 9. Kinetics of the Degradation of Poly(C) and Poly(U) by RNase A in the Presence and Absence of Spermine

The assays were carried out under standard conditions except that the amounts of RNase A used for the degradation of poly(C) [A] and poly(U) [B] were 1.5 and 12 ng, respectively, and the reaction mixture contained various amounts of substrate and spermine as specified in the figure. The average molecular weight of polynucleotides used was 60000.
●—●, no spermine; ○—○, 0.04 mM spermine.

(C), and poly(U) were 1.60, 2.29, 1.90, and 3.08 mM, respectively. However, spermine increased the maximal velocity of the reaction, except when poly(U) was used as a substrate. For example, the increases in typical experiments were as follows: RNase activity increased from 0.38 to 0.98 nmol/min in 2′,3′-cCMP hydrolysis (0.02 mM spermine), from 0.8 to 1.2 nmol/min in 2′,3′-cUMP hydrolysis (0.02 mM spermine), and from 8.0 to 10.8 nmol/min in poly(C) degradation (0.04 mM spermine). The maximal velocity of poly(U) degradation was about 16.0 nmol/min in the presence and absence of 0.04 mM spermine.

Discussion

The data presented here show that the hydrolyses of all of the tested cyclic nucleotides and benzyl esters of nucleotides by RNase A were stimulated by polyamines. These effects of polyamines on the hydrolysis of low molecular weight nucleotides are in contrast to previous results which showed that polyamines markedly stimulated the degradation of poly (C) but slightly inhibited the deradation of poly (U).[4] It is of interest to know how many nucleotide moieties are necessary to modify the effect of polyamines on the enzymatic cleavage of $C_{5'}$-O-P linkages adjacent to a uracil nucleotide in oligonucleotide molecules from stimulation to inhibition. Recently, Dr. M. Irie of Hoshi College of Pharmacy, Tokyo, examined the effect of spermine on the RNase A hydrolysis of uridylyl-(3′-5′)-ribonucleosides and observed slight inhibition by spermine.[11] These results suggest that at least two nucleoside moieties are necessary for polyamines to inhibit the cleavage of $C_{5'}$-O-P linkages adjacent to a uracil nucleotide.

The experimental data (Figs. 8 and 9) clearly show that polyamines did not significantly change the apparent *K*m of the enzyme for the substrates[2′,3′-cCMP, 2′,3′-cUMP, poly(C), and poly(U)], but increased the maximal velocity of the reaction, except when poly(U) was

11) M. Irie, personal communication.

used as a substrate. In addition, the optimal concentration of spermine for stimulation increased as the substrate concentration increased. These results suggest that polyamines do not interact with the enzyme, but probably act *via* binding to the substrates.

The binding of polyamines to single-stranded polynucleotides was found to be in the order poly(U)>poly(C)>poly(A),[12] but the binding of polyamines to different nucleoside diphosphates was nearly equal.[13] This may explain why spermine changed the base specificity of RNase A more with homopolynucleotides than with cyclic nucleotides or benzyl esters of 3′-nucleotides.

It is of interest that NH_4^+ and K^+ had an effect similar to that of polyamines on the hydrolysis of cyclic nucleotides and homopolynucleotides by RNase A (Fig. 7). Functional similarities of polyamines and NH_4^+ (or K^+) ions have been observed in the binding of these ions to single-stranded synthetic polynucleotides,[12] the breakdown of synthetic polynuleotides by *Escherichia coli* RNase II,[14] and the amino acid-dependent pyrophosphate-ATP exchange catalyzed by aminoacyl-tRNA synthetase.[15]

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12) K. Igarashi, Y. Aoki, and S. Hirose, *J. Biochem.* **81**, 1091 (1977).
13) C. Nakai and W. Glinsmann, *Biochemistry*, **16**, 5636 (1977).
14) H. Kumagai, K. Igarashi, M. Yoshikawa, and S. Hirose, *J. Biochem.*, **81**, 381 (1977).
15) I. Svensson, *Biochim. Biophys. Acta*, **146**, 239 (1967); K. Igarashi, K. Matsuzaki, and Y. Takeda, *ibid*, **254**, 91 (1971); A. Pastuszyn and R.B. Loftfield, *Biochem. Biophys. Res. Commun.*, **47**, 775 (1972).